AD-A193 676 MOLECULAR BIOLOGY OF THE EXTREMELY THERMOPHILIC ARCHAEBACTERIUM METHANOTHERMUS FERVIDUS(U) OHIO STATE UNIV COLUMBUS DEPT OF MICROBIOLOGY J N REEVE 15 APR 88 1/1

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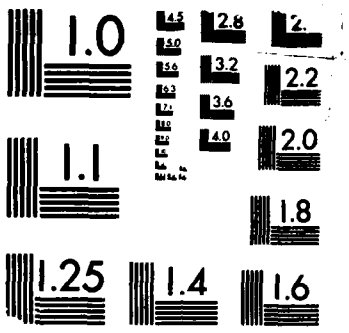

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Molecular Biology of the extremely thermophilic archaebacterium, Methanothermus fervidus

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Genes encoding the subunit polypeptides of methyl coenzyme M reductase, tRNAs and rRNAs have been cloned and sequenced from the extremely thermophilic archaebacterium Methanothermus fervidus. The primary sequences of these genes have been compared with the functionally equivalent sequences from mesophilic and less thermophilic methanogens. Highly conserved regions and structural features which may play a role in thermostability have been identified. A DNA binding protein (HMf) has been purified from M. fervidus which, when bound to double-stranded DNA, increases the temperature of its thermal denaturation, in vitro, by as much as 40° C.

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Annual Report: Contract N00014-86-K-02111

Principal Investigator: John N. Reeve

Contractor: The Ohio State University

Contract Title: Molecular Biology of the Extremely Thermophilic Archaebacterium, Methanothermus fervidus.

Period of Performance: 4/1/87 - 3/21/88

Research Objectives

The goals of our research are to determine the structure and mechanisms of regulation of expression of protein and stable RNA encoding genes in M. fervidus, to investigate the role of DNA binding proteins in the structure and expression of the M. fervidus genome and to analyse the structure of tRNA, rRNAs and polypeptides for features which provide a basis for their thermostabilites.

Progress

Methyl coenzyme M reductase

The most abundant enzyme in methanogens, methyl coenzyme M reductase, which catalyzes the terminal reaction in methanogenesis has been chosen as the subject for detailed investigation in M. fervidus. The function and subunit structure ($\alpha_2\beta_2\gamma_2$) of this enzyme appears to be consereved in all methanogens and therefore changes in the primary structure of the α, β and γ subunits can be used to estimate evolutionary divergences and may also indicate changes which have occured to accommodate different environments. We had previously cloned and sequenced the genes, mcrBDCGA, which encode methyl reductase in Methanococcus vannielii and have now used the mcrA gene from M. vannielii as a hybridization probe to clone the methyl reductase operon from M. fervidus. The organization of mcr genes in M. fervidus has been found to be the same as in M. vannielii (1), Methanosarcina barkeri (2) and Methanobacterium thermoautotrophicum (3). There are five closely linked open-reading frames mcrBDCGA. The α, β and γ subunits of methyl reductase are encoded by mcrA, mcrB and mcrG respectively and two genes mcrD and mcrC which encode unknown products separate mcrB and mcrG. The complete sequence (6Kbp) of the mcr operon in M. fervidus has been determined, plus upstream and downstream regions (Figure 1), and compared with the sequences from M. vannielii, M. barkeri and M. thermoautotrophicum. As shown in Figure 2 the polypeptide encoded by the mcr genes are conserved in all four methanogens. The most closely related sequences are those from the two thermophiles, M. fervidus and M. thermoautotrophicum. The mcrD genes and gene products have diverged to the greatest extents.

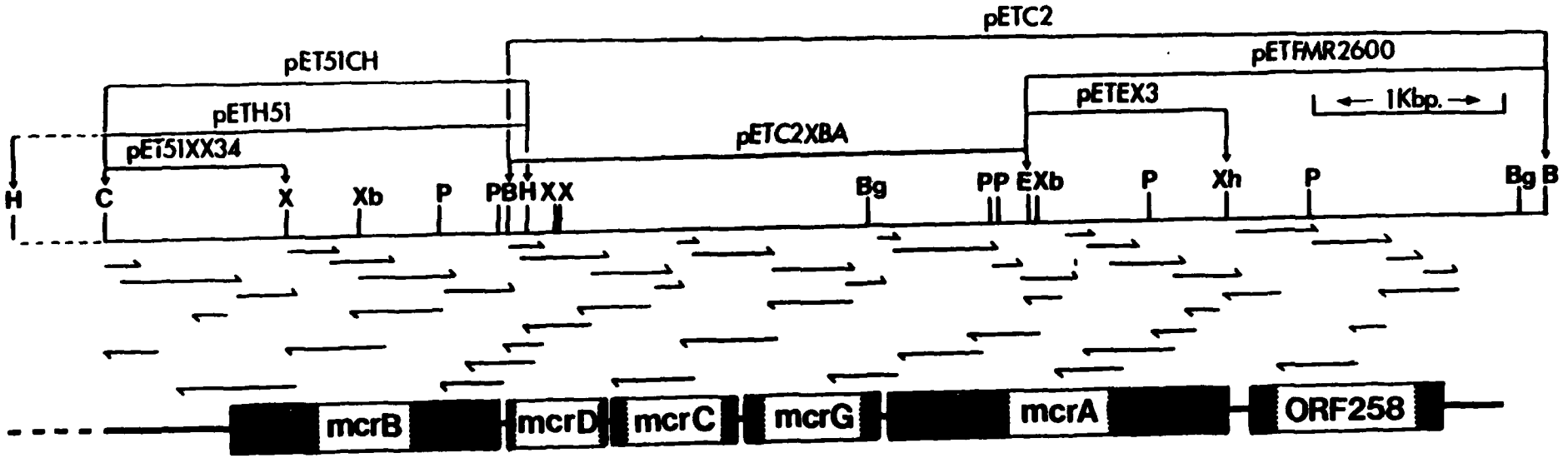

Figure 1. Organization, subcloning and sequencing strategy of the mcr gene cluster from M. fervidus. The regions of the M. fervidus genome cloned in pUC9-and pUC18-based plasmids are indicated below the plasmid (pET) designations. Restriction sites are indicated as B, BamH1; Bg, BglII; C, ClaI; E, EcoRI; H, HindIII; P, PstI; X, XmnI; Xb, XbaI and Xh, XhoI. The individual regions and direction of their sequencing are shown by the arrows below the restriction map. The α, β and γ subunits of methyl reductase are encoded by the boxed genes designated mcrA, mcrB and mcrG respectively. The products of the mcrD, mcrC and ORF258 genes have yet to be identified.

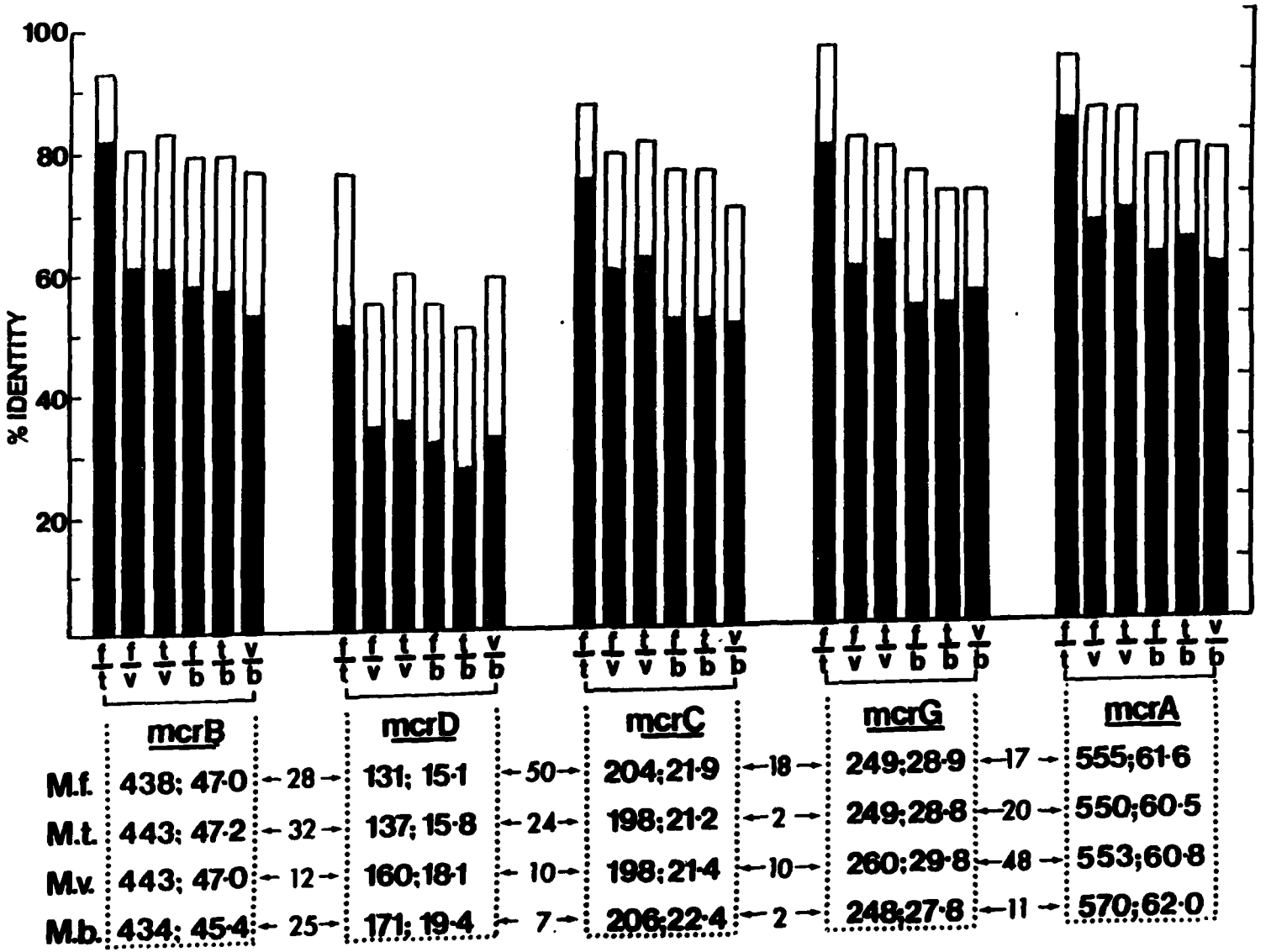

Figure 2. Comparison of mcr gene products. The percentages of identical amino acids in a pair of polypeptides is indicated by the shaded box and the increase in this value which results from considering amino acid substitutions which maintain approximate size, charge and hydrophobicity as maintaining identity, is indicated by the open box. The polypeptides compared are designated f from M. fervidus; t from M. thermoautotrophicum (2); v from M. vannielii (1); and b from M. barkeri (3) above the encoding gene. The values below the gene are the numbers of amino acid residues and calculated molecular weights (in Kdal) of the gene products. The number of base pairs which form intergenic regions are listed between the arrows at their locations within the mcr gene clusters of M. fervidus (M.f.), M. thermoautotrophicum (M.t.), M. vannielii (M.v.) and M. barkeri (M.b.).

Preliminary analyses of the sequences of the polypeptides encoded in M. fervidus by the mcr genes do not indicate obvious correlations with thermophily. More detailed computer assisted analyses of potential secondary structures of mRNAs and polypeptides need to be undertaken. Very conserved regions, within the methyl reductase subunits, have been identified which are presumably the active sites or cofactor binding sites for the enzyme. These would be ideal candidate sequences for site-specific mutagenesis to investigate the detailed mechanism of catalysis by methyl reductase. The mcr genes in M. fervidus are preceded by extended ribosome binding sites but the sequence identified as the promoter for transcription of the mcr operons in M. vannielii (4) is not conserved in M. fervidus. Similarly sequences downstream of the mcr operon in M. vannielii (1) and M. thermoautotrophicum (2) which appear to function as transcription terminators are not present in the M. fervidus sequence. The open reading frame (ORF258) 3' to mcrA in M. fervidus (Figure 1) encodes an amino acid sequence which is 40% identical to the sequence of amino acids encoded by a truncated ORF identified at this location in M. thermoautotrophicum (2).

Overall the results to date obtained from the mcr analyses demonstrate that although the polypeptide encoding regions are highly conserved the intergenic regions show little conservation. Promoter structures and regulatory signals appear to have diverged much more than the enzyme encoding sequences.

Stable RNA encoding genes

Clusters of stable RNA encoding genes have been cloned from gene banks by hybridization with the end-labeled, mature RNAs. Two of these clusters which code for tRNAs and one which encodes rRNAs have been sequenced. The genomic organizations of these tRNA genes in M. fervidus are similar to the organizations found in M. vannielii and M. voltae (5) but, unlike the methanococci, the M. fervidus tRNA clusters do not contain 5SrRNA encoding genes. Genes encoding the same $tRNA^{tr}$, $tRNA^{pro}$, $tRNA^{asp}$ and $tRNA^{lys}$ are linked in M. fervidus, M. vannielii and M. voltae but the methanoccal genomes also contain 5SrRNA genes and additional tRNA genes (Figure 3). A second cluster of tRNA genes is exactly conserved in M. fervidus and M. vannielii (Figure 3). The sequences of these tRNA genes have been compared. Whereas the sequences of the M. vannielii and M. voltae genes are virtually identical, the M. fervidus sequences are only 80-90% identical to these methanococcal sequences. Although genomic organizations has been conserved, primary sequences have apparently diverged to a significant extent (Table 1). The tRNA sequences have been analyzed for their ability to form stable double-stranded RNA (dsRNA) regions; it is clear that the tRNAs from M. fervidus have increased opportunities to form G:C base-pairs in dsRNA stem structures. None of the sequenced M. fervidus tRNA genes contain introns.

Southern hybridization experiments using rRNA probes and a range of restriction enzyme digests of M. fervidus genomic DNA have demonstrated that there are two clusters of linked 16S-23S-5S rRNA encoding genes in M. fervidus. We have cloned both these clusters and have sequenced the 5S rRNA gene and part of the 16SrRNA gene from one of these clusters. The 5SrRNA shows features typical of all archaebacteria and is most closely related to

Figure 3. Comparison of tRNA clusters. The organization of tRNA and 5S rRNA genes in M. fervidus, M. vannielii and M. voltae (5).

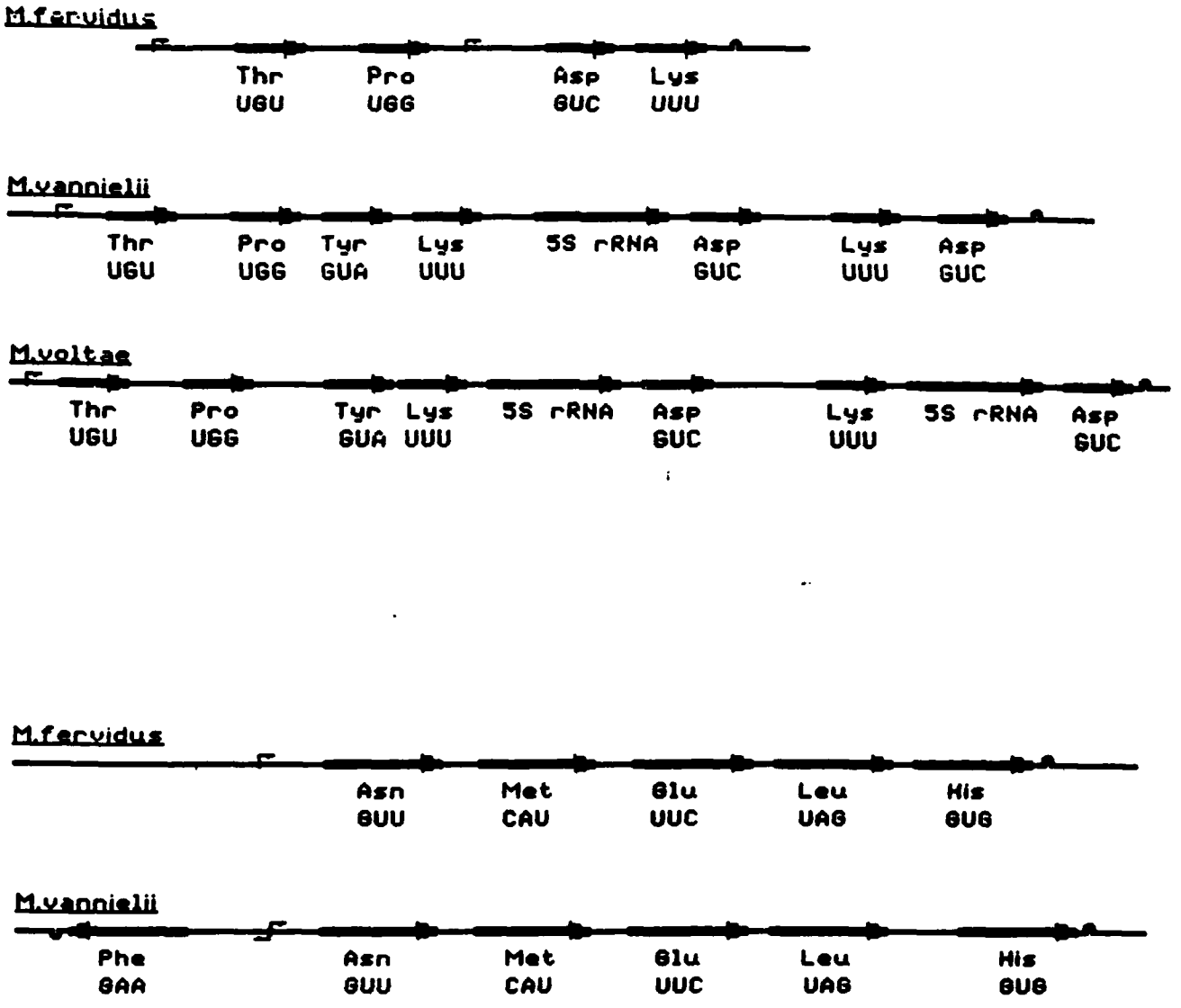

Table 1. Percentages of identical bases in tRNAs.

	Thr UGU	Pro UGG	Asp GUC	Lys UUU	Asn GUU	Met CAU	Glu UUC	Leu UAG	His GUG	AVG
Mf vs Mv	88	82	83	86	81	84	89	69	78	82
Mf vs Hv	NA	80	79	77	81	79	84	67	72	77
Mv vs Hv	NA	77	77	78	71	73	84	71	74	76
Mf vs Mvo	84	75	83	81	NA	NA	NA	NA	NA	81
Mvo vs Mv	96	96	100	97	NA	NA	NA	NA	NA	97
Mvo vs Hv	NA	74	77	82	NA	NA	NA	NA	NA	77

Abbreviations: Mf = Methanothermus fervidus
Mv = Methanococcus vannielii
Mvo = Methanococcus voltae
Hv = Halobacterium volcanii
NA = not available

the 5SrRNA of M. thermoautotrophicum (6). There are extensive regions of dsRNA which are rich in G:C pairs. This is in contrast to the overall low (~31%) G+C content of the genome of M. fervidus.

Sequences flanking the tRNA and rRNA encoding genes have been analyzed for conserved, potential promoter sequences. Sequences, at the expected locations, have been identified which are very similar to sequences already proposed as promoters for transcription of tRNA gene in methanococcal species (7). Sequences likely to cause transcription termination cannot, however, readily be identified.

DNA binding proteins

Purified M. fervidus DNA denatures at 83° in vitro so that it was reasonable to presume that DNA binding protein(s) must be present within M. fervidus cells which prevent DNA denaturation in vivo. We have now purified, to homogeneity, a small (~10Kdal) DNA-binding protein (designated HMf) from M. fervidus which when bound to dsDNA in vitro increases the thermal denaturation temperature of the DNA by 40°. HMf is present in large amounts (~ 1% of the cytoplasmic protein) and can be assayed by its ability to increase the electrophoretic mobility of fragments of dsDNA through agarose gels. Binding of HMf to DNA is non-covalent, occurs at all tempertures and does not appear to have sequence specificity. Saturation of DNA molecules with HMf indicates that the polypeptide probably binds as a oligomeric protein to approximately 40 bp of DNA per oligomer.

Citations

(1) Cram D.S., B.A. Sherf, R.T. Libby, P.J. Mattaliano, K.L. Ramachandran and J.N. Reeve. 1987. Proc. Natl. Acad. Sci. U.S.A. 84:3992-3996.

(2) Bokranz M., G. Baumer, R. Allmansberger, D. Ankel-Fuchs, and A. Klein. 1988. J. Bacteriol. 170:568-577.

(3) Bokranz M. and A. Klein. 1987. Nucl. Acad. Res. 15:4350-4362.

(4) Thomm M., B.A. Sherf and J.N. Reeve. 1988. J. Bacteriol. 170:1958-1961.

(5) Wich G., M. Jarsch and A. Böck. 1984. Mol. Gen. Genet. 196:146-151.

(6) Wolters J. and V.A. Erchmann. 1986. J. Mol. Evol. 24:152-166.

(7) Wich G., H. Hummel, M. Jarsch, V. Bar and A. Bock. 1986. Nucl. Acid. Res. 14:2459-2479.

Objectives for the next year.

1) The primary transcripts of the *mcr* operon and clusters of stable RNA genes will be characterized. Stability and processing of these RNA molecules will be determined.

2) DNA-dependent-RNA polymerase will be purified from *M. fervidus* and used to define promoter sequences upstream of the *mcr* operon and the stable RNA genes. Footprinting techniques for use with this thermophilic enzyme will be developed. We anticipate that these experiments may require an extensive commitment of time and effort. Assays at the growth temperature (~80°) of *M. fervidus* will be required for fully meaningful results.

3) The interaction of HMf with dsDNA molecules will be characterized by electron microscopy and in competition assays using alternative DNA substrates. The effects of HMf on the activities of DNA polymerase, RNA polymerase and topoisomerases *in vitro* will be determined.

4) Extensive analyses will be made of predicted secondary structures of *M. fervidus* RNAs and polypeptides will be undertaken based on the available primary sequences. Addition and sequencing of tRNA and rRNA genes will be completed.

Inventions: None

Publications:

The following publications acknowledge ONR support:

1. Reeve, J.N., Beckler, G.S., Brown, J.W., Cram, D.S., Haas, E.S., Hamilton,

P.T., Morris, C.J. Sherf, B.A. and Weil, C.F. 1987. Divergence of methanogens, conservation of the hisI gene sequence in all three biological kingdoms and the status of M. thermoautotrophicum. In: Proc. Fifth International Symposium on Microbial Growth on C1-Compounds. ed. van Verseveld, H.W. and Duine, J.A. pp. 255-260.

2. Reeve, J.N., Beckler, G.S. and Cram, D.S. 1987. Methanogens are archaebacteria; similarities to and differences from the molecular biology of eubacteria and eucaryotes. Proc. Fifth Internation Syposium on the Genetics of Industrial Microorganisms. pp. 303-311.

3. Reeve, J.N. 1987. Gene structure in methanogenic bacteria. Poultry Sci. 66:927-933.

4. Weil, C.F., Beckler, G.S. and Reeve, J.N. 1987. Structure and organization of the hisA gene of the thermophilic archaebacterium Methanococcus thermolithotrophicus. J. Bacteriol. 169:4857-4860.

5. Reeve, J.N., (1987) Methane Genes. In "Anaerobes Today" eds. Hardie J.E. and Boriello, S.P. John Wiley Publishing Inc. Shrewsburg England. In Press.

6. Morris C.J. and Reeve, J.N. 1988. Conservation of structure in the human gene encoding arginino-succinate synthetase and the argG genes of the archaebacteria Methanosarcina barkeri and Methanococcus vannielii. J. Bacteriol. 170: In Press.

7. Weil C.F., Cram D.S. and Reeve J.N. 1988. Structure and comparative analysis of the genes encoding component C of methyl coenzyme M reductase in the extremely thermophilic archaebacterium Methanothermus fervidus. J. Bacteriol. Submitted for publication 4/88.

Abstracts

Krzycki J.A., Timans C.J. and Reeve J.N. 1988. A DNA-thermostabilizing and compacting protein from Methanothermus fervidus. Abst (1988) Annual Meeting of the American Society for Microbiology.

Invited Research Presentation:

The P.I. has given the following presentations:

4/6/87: University of Oklahoma, Norman, OK. Guest of the Department of Botany & Microbiology. Research Seminar: "Archaebacterial Molecular Biology".

4/16/87: Kenyon College, Gambier, OH. Guest of the Biology Department. Research Seminar: "Microorganisms in Extreme Environments".

5/12/87: University of Georgia, Athens, GA. Guest selected by the Graduate Students, Dept. of Microbiology. Review Seminar: "Molecular Biology of Archaebacteria".

6/29/87: Gordon Conference on Methanogens, Tilton, New Hampshire. Invited Symposium Lecturer: "Methane Genes".

7/24/87: Fifth International Symposium on Anaerobes. Cambridge, England. Invited Symposium Lecturer: "Methane Genes".

8/4/87: University of Sophia, Bulgaria. Guest of the Bulgarian Academy of Sciences. Research Seminar: "Biotechnology and Archaebacteria".

10/10/87: University of Wyoming, Laramie, WY. A.S.M. Foundation Lecture "Molecular Biology of Archaebacteria". Guest of the Rocky Mountain Branch of the American Society for Microbiology.

11/6/87: University of Southwestern Louisiana, Hammond, LA. A.S.M. Foundation Lecture: "Methanogen and Methane Genes", Guest of the S. Central Branch of the American Society for Microbiology.

11/13/87: Joint Meeting of the Florida, Kentucky and S.E. Branchs of the A.S.M., Orlando, Fl. Foundation Lecture: "Methane Genes". Guest of the Florida Branch of the American Society for Microbiology.

3/11/88: Rensselaer Polytechnic Institute, Troy, N.Y. A.S.M. Foundation Lecture "Methane Genes and the Molecular Biology of Methanogens." Guest of the Eastern N.Y. Branch of the American Society for Microbiology.

Personnel:

ONR funds have been used directly as salary support for Dr. C.F. Weil who completed the study of the M. thermolithotrophicus hisA gene, cloned and sequenced the mcr genes of M. fervidus.

Research undertaken by Dr. J. Krzycki and two undergraduate students, Mr. P. Soloman and Mr. C. Timan resulted in the identification and isolation of the DNA binding protein. Their work and that of Ms. E. Haas, the senior graduate student who cloned and sequenced the M. fervidus 5S rRNA and tRNA genes, was made possible by using ONR funds to purchase small equipment, media and consumable supplies.

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